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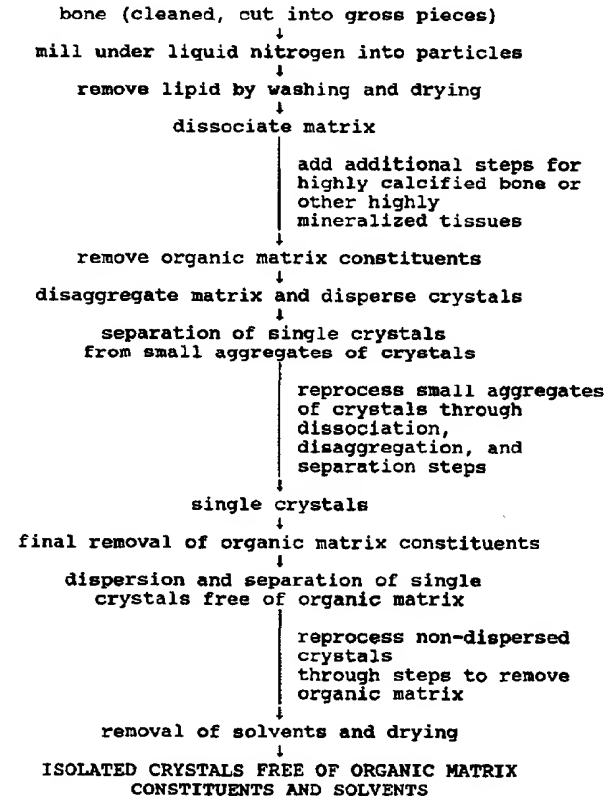
(57) Abstract

The present invention is a process for first removing and isolating the calcium-phosphate crystals of bone from a substantial amount of the organic matrix and cellular constituents of bone without significant physical, chemical or structural alterations in the crystals. The crystals can then be further treated to remove the remaining amount of organic material associated with the crystals, leaving them essentially free of any of the organic constituents of bone, without significant physical, chemical, or structural alterations in the crystals.



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ISOLATION OF THE CALCIUM-PHOSPHATE CRYSTALS OF BONE

Background of the Invention

The present invention is generally in the area of purification of the naturally produced biological apatite crystals of bone, and of highly purified, calcium-phosphate apatite crystals of bone produced by these methods.

Calcium hydroxyapatites occur naturally as geological deposits and in normal biological tissues, principally bone, cartilage, enamel, dentin, and cementum of vertebrates and in many sites of pathological calcifications such as blood vessels and skin. Synthetic calcium hydroxyapatite is formed in the laboratory either as pure $Ca_{10}(PO_4)_6(OH)_2$ or hydroxyapatite that is impure, containing other ions such as carbonate, fluoride, chloride for example, or crystals deficient in calcium or crystals in which calcium is partly or completely replaced by other ions such as barium, strontium and lead. Essentially none of the geological and biological apatites are "pure" hydroxyapatite since they contain a variety of other ions and cations and may have different ratios of calcium to phosphorous than the pure synthetic apatites. In general, the crystals of pure synthetic apatites, geological apatites and many impure synthetically produced apatites are larger and more crystalline than the biological crystals of bone, dentin, cementum and cartilage.

The calcium-phosphate (Ca-P) crystals of the bones of essentially all vertebrates have the basic crystal structure of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] as determined by x-ray diffraction. Indeed, the calcium-phosphate (Ca-P) crystals of essentially all of the normally mineralized tissues of vertebrates, including enamel, dentin, cementum, and calcified cartilage, have the same general crystal structure. There are few exceptions, notably the enamel of shark teeth

which have fluoride ions substituted for many of the hydroxyl groups.

However, the crystals of Ca-P found in biological tissues such as bone also contain other atoms and ions such as acid phosphate groups (HPO_4^{-2}), and carbonate ions (CO_3^{-2}), which do not occur in pure, synthetic hydroxyapatite. There is also good evidence that bone crystals either do not contain hydroxyl groups, or contain only very few such groups (Bonar, et al., "Structural and composition studies on the mineral of newly formed dental enamel: a chemical, x-ray diffraction, and ^{31}P and proton nuclear magnetic resonance study" *J. Bone Min. Res.* 6:1167-1176 (1991), and is therefore more appropriately referred to as "apatite" rather than "hydroxyapatite". Moreover, many of the carbonate and phosphate groups in bone crystals are, from the structural and physical chemical points of view, unstable and very reactive, thus providing certain physical chemical and biological functional and chemical features important in the formation and dissolution of the crystals in biological tissues.

Recent important ^{31}P-nuclear magnetic resonance spectroscopy studies have also demonstrated that the short-range order or environment of the HPO_4^{-2} groups in bone crystals are distinctly different than the HPO_4^{-2} groups in synthetic apatites and other related calcium-phosphate crystals (Wu, Ph.D. thesis M.I.T., "Solid state NMR study of bone mineral", August, 1992). These differences in chemical, structural, and short range order of the bone crystals compared with pure, synthetic hydroxyapatite also reflect significant differences in their reactivity and hence in their potential function in a biological environment.

The crystals of bone, dentin and cementum are very small, irregularly shaped, very thin plates whose

rough average dimensions are approximately 10 to 50 angstroms in thickness, 30 to 150 angstroms in width, and 200 to 600 angstroms in length. This results in their having a very large surface area to present to the extracellular fluids which is critically important for the rapid exchange of ions with the extracellular fluids. This "ion-reservoir" function of the inorganic crystals is very important for a number of critical biological functions.

The vast majority of the Ca-P crystals of bone are located within the collagen fibrils of bone, as reported by Glimcher, M.J., "A basic architectural principle in the organization of mineralized tissues" In: Milhaud, A.G., ed. Proceedings of the Fifth European Symposium on Calcified Tissues, Bordeaux, France, 1968, Lee and Glimcher, "Three-dimensional spatial relationship between the collagen fibrils and the inorganic calcium phosphate crystals of pickerel (Americanus americanus) and herring (Clupea harengus) bone", J. Mol. Biol. 217: 487-501 (1991); and Glimcher MJ, "Molecular biology of mineralized tissues with particular reference to bone" Rev. Mod. Physics 31:359-393 (1959). In general, bone contains approximately 35% organic constituents, the major component being collagen fibrils. See, for example, Cohen-Solal, et al., "Identification of organic phosphorus covalently bound to collagen and non-collagenous proteins of chicken-bone matrix: the presence of O-phosphoserine and O-phosphothreonine in non-collagenous proteins, and their absence from phosphorylated collagen" Biochem. J. 177:81-98 (1979). Due to their intimate physical location and interrelationship with the collagen fibrils, it has not heretofore been possible to separate and isolate the crystals of bone from the collagen fibrils of bone and other organic constituents of the tissue without producing significant changes in the chemistry,

structure, degree of crystallinity and size of the crystals, as reported by Sakae, et al., "Changes in bovine dentin mineral with sodium hypochlorite treatment, J. Dental Res. 1229-1234 (1988).

Methods previously used to remove and isolate the calcium-phosphate apatite crystals of bone have not been successful, either because they do not completely separate the crystals from the organic constituents and/or because they alter the chemistry and structure of the crystals. For example, hydrazine treatment of well mineralized bone carried out at temperatures of 50°C and higher yielded crystals containing significant amounts of organic constituents and induced significant changes in the crystals. Similarly, while substances such as sodium hypochlorite released calcium-phosphate apatite crystals from bone and other tissues, it was used in the form of an aqueous solution. Contact of bone crystals with water for even short periods of time has been shown to significantly alter the crystals by dissolution, reorganization, re-precipitation, and cannot be prevented by adding calcium and phosphate ions to the water based solution. See, for example, Landis, et al., "Electron microscopic observations of bone tissues prepared by ultracryomicrotomy" J. Ultrastruct. Res. 59:185-206 (1977); Landis, et al., "Electron microscopic observations of bone tissue prepared anhydrously in organic solvents" J. Ultrastruct. Res. 59:1-30 (1977); and Landis, et al., "Electron diffraction and electron probe microanalysis of the mineral phase of bone tissue prepared by anhydrous techniques" J. Ultrastruct. Res. 63:188-223 (1978)Furthermore, it has been found that the crystals are not only altered but also contains significant amounts of organic matrix. In a similar fashion, plasma ashing of bone to remove the organic matrix and disperse the crystals has been shown to induce major

alterations in the crystal which as in the other methods described above can also contain significant amounts of organic constituents. Such treatment seriously alters the chemistry and structure of the crystals.

The synthetic materials are highly diverse, as reported in the literature. For example, the characterization of four commercial apatites was reported by Pinholt, et al., J. Oral Maxillofac. Surg. 50(8), 859-867 (August 1992); J. Cariofac. Surg. 1(3), 154-160 (July 1990) reports on a protein, biodegradable material; Pinholt, et al., Scand. J. Dent. Res. 99(2), 154-161 (April 1991) reports on the use of a bovine bone material called Bio-Oss™; Friedman, et al., Arch. Otolaryngol. Head Neck Surg. 117(4), 386-389 (April 1991) and Costantino, et al., Arch. Otolaryngol. Head Neck Surg. 117(4), 379-384 (April 1991) report on a hydroxyapatite cement; Roesgen, Unfallchirurgie 16(5), 258-265 (October 1990), reports on the use of calcium phosphate ceramics in combination with atogeneic bone; Ono, et al., Biomaterials 11(4), 265-271 (May 1990) reports on the use of apatite-wollastonite containing glass ceramic granules, hydroxyapatite granules, and alumina granules; Passuti, et al., Clin. Orthop. 248, 169-176 (Nov. 1989) reports on macroporous calcium phosphate ceramic performance; Harada, Shikwa-Gakuho 89(2), 263-297 (1989) reports on the use of a mixture of hydroxyapatite particles and tricalcium phosphate powder for bone implantation; Ohgushi, et al., Acta Orthop. Scand. 60(3), 334-339 (1989) reports on the use of porous calcium phosphate ceramics alone and in combination with bone marrow cells; Pochon, et al., Z-Kinderchir. 41(3), 171-173 (1986) reports on the use of beta-tricalcium phosphate for implantation; and Glowacki, et al., Clin. Plast. Surg. 12(2), 233-241 (1985), reports on the use of demineralized bone

implants. No general conclusions can be drawn from these representative reports except that the need for materials which are useful in fixation of implants and in repair or replacement of bone defects remains and that the materials now available do not solve the many problems associated with the treatment of these problems, due to many variables, including the properties of the materials as well as the ease with which they can be manufactured and utilized by the surgeon.

The majority of synthetic hydroxyapatite preparations that have been proposed for use as bone inductors (to induce bone formation) and osteoconductors (by acting as scaffolds to facilitate for the continuous progression of new bone formation) are of synthetic origin and distinct structurally and chemically from the biological calcium-phosphate crystals in bone. All of these apatites are not only chemically and structurally distinct from the apatite crystals of bone, especially in their short range order, size and reactivity, but in some cases, they contain varying amounts of amorphous calcium-phosphate, that is, calcium-phosphate solids which are not crystalline at all. In other instances, the calcium-phosphates made synthetically also contain calcium salts other than apatite crystals such as calcium oxides. To date, it has not been shown how these additional calcium salts are biocompatible or without untoward effects, either biologically or structurally, nor how they affect the bonding strength between the synthetic apatites used to coat the surfaces of artificial joints implanted to bone and the surface of the artificial joint, and between the synthetic apatites and the bone into which the device is implanted.

It is therefore an object of the present invention to provide the biologically, naturally

formed crystals of bone a purified apatite that are substantially free of organic material but which also consist predominantly of highly uniform crystals with respect to the chemistry, structure, size, shape and index of crystallinity.

It is a further object of the present invention to provide methods for the further purification of bone apatite crystals that remove essentially all organic material without disrupting the natural crystalline structure of the bone crystals.

Summary of the Invention

The present invention is a process for removing and isolating the calcium-phosphate crystals of bone from a substantial amount of the organic matrix and cellular constituents of bone without significant physical, chemical or structural alterations in the crystals isolated from bone, cementum, dentin, enamel, and cartilage (referred to collectively herein as "bone"). The crystals can then be further treated to remove the remaining amount of organic material associated with the crystals, leaving them essentially free of any of the organic constituents of bone, without significant physical, chemical or structural alterations in the crystals, that is, having the same chemical composition, structure, short range order (as measured using standard techniques), and index of crystallinity as the native bone from which it is derived.

The calcium-phosphate (Ca-P) crystals (also referred to as "apatite") of bone are first isolated and purified by performing an initial separation to separate the crystals from the highly dense connective tissue in bone, then by reacting the dried bone powder with a polar solvent such as hydrazine at low temperatures. In young, poorly mineralized bone, the hydrazine step can be performed on the bone powder

directly following grinding and milling at low temperatures. However, with more mature bone, the bone powder is first dispersed in a solvent such as ethanol, in the complete absence of water, and sonicated to achieve a gentle separation of organic matrix from bone crystals, then the crystals can be treated with hydrazine and/or plasma ashing at low temperatures. This process separates the crystals from the collagen fibrils and bundles of fibrils making up the densely packed tissue matrix, leaving the crystals relatively well dispersed and isolated from each other so that they are more readily accessible to chemical and/or physical techniques used to remove the remaining organic constituents without altering the crystal structure or chemistry. In either process, the bone particles can first be treated with plasma ashing.

Key aspects of the processes are that they do not utilize aqueous conditions at any point, they are performed at low temperatures, and they do not include any techniques inducing changes in the crystal structure or chemistry, such as high power sonication or grinding at warm temperatures.

These procedures yield isolated crystals free of collagen fibrils even from adult, heavily calcified, mature bone. The crystals have the signature characteristics of bone crystals in native bone, both analytically, chemically and structurally. Transmission electron microscopy reveals well dispersed crystals, free of any observable collagen fibrils or any other organic material. Chemical analysis, x-ray diffraction, and Fourier transform infrared spectroscopy show the typical characteristics of the crystals in native bone, including the presence of carbonate and acid phosphate groups, and the absence of hydroxyl groups as detected by Fourier

transform infrared spectroscopy and proton nuclear magnetic resonance spectroscopy.

The isolated calcium-phosphate crystals are useful in a variety of applications, including chromatographic separation and isolation of proteins and in medical or therapeutic applications, such as in the healing and repair of bone, the replacement of bone with the eventual formation of new bone in the defects, and, in general, in the induction of new bone and in the osteoconductive progression of new bone formation, including the coating of specific surfaces of artificial joints or teeth implanted in bone.

Brief Description of the Drawing

Figure 1 is an abbreviated schematic flow chart of a general method for the separation and isolation of calcium-phosphate crystals from young, poorly mineralized bone (I) or more mature, more heavily mineralized bone (II).

Detailed Description of the Invention

As used herein, "purified" and "purification" are not terms which are used to suggest that the apatite crystals are synthesized *de novo in vitro*; rather they refer to procedures to remove, disperse and isolate the natural, native, biological crystals which are in bone from bone, cementum, enamel, dentin and cartilage, without significantly altering their physical shape, size, structure or chemistry. They can be prepared so that their dry weight contains roughly as little as 25% or less of the total dry weight as organic matrix constituents or be further subjected to procedures which remove more of the organic constituents so that as little as approximately 2% or less of their dry weight is accounted for by organic constituents.

The exact determination of the size and habit of the extremely small calcium-phosphate (Ca-P) crystals in bone, and their short range order and fine structure, has been hampered because the crystals are embedded principally within collagen fibrils, which themselves are densely packed into fibers and fiber bundles of the highly organized extracellular matrix of the tissue fabric. Previous attempts to isolate the crystals free of the collagen fibrils and other organic matrix constituents by reaction with hydrazine and other reagents or by plasma ashing have left variable but significant quantities of organic matrix in the samples and have produced readily detectable and significant changes in the crystals.

Bone mineral crystals are isolated by a combination of a series of techniques which disaggregate the bone matrix and facilitate additional methods which remove the organic matrix constituents, and which permit the dispersion of single crystals and small aggregates of crystals. The final result of these processes yields the native bone crystals free of organic matrix constituents. All steps in the procedure are performed in non-aqueous conditions and at low temperature: dissociation of matrix at less than 36°C, removal of matrix at between 2 and 4°C with hydrazine, plasma ashing at 0°C or lower, and dispersion and separation of the crystals at less than 10°C. The methods described herein are shown schematically in Figure 1, where (I) is the preferred process for use with young, not highly mineralized bone, and (II) is the preferred processes for use with more mature, and more mineralized bone. A combination of several of the above techniques can also be used depending on the level of mineralization of the tissue, the density of packing in the tissue, and other variables.

Isolation and characterization of Calcium-phosphate crystals from Bone.

A method has been developed to remove and isolate the calcium-phosphate apatite crystals from bone that yields calcium-phosphate crystals that are essentially completely free of organic material and which have been shown not to have been significantly altered with regard to their structure or chemistry. As used herein, "bone" includes other biological sources of calcium-phosphate crystals including dentin, enamel, cementum and cartilage. "Purified" means separating and isolating the naturally occurring biological crystals in bone away from the organic matrix constituents, especially collagen fibrils, to less than 25%, most preferably less than 1%, of their total dry weight. The same procedures can be applied to other biologically calcified tissues such as the exoskeletons of invertebrates like coral which contain crystals of calcium carbonate. This material has also been used in combination with repair or replacement of bone.

The initial separation of the crystals of bone is accomplished by dissociation of bone matrix constituents; decomposition of organic matrix constituents; disaggregation of dissociated bone matrix and the decomposed organic matrix; dispersion of single crystals and small aggregates of crystals; separation of single crystals; and removal of solvents. Importantly, none of the reagents or procedures used in processing of the crystals can include water. This is in contrast with other reported methods, for example, using sodium hypochlorite as a water based solution.

a. Preparation of bone particles

Bone powder is prepared by mill grinding fresh bone in liquid nitrogen and sieving to a particle size ranging up to approximately 200 microns, preferably 75 to 200 microns. The exact total times for mill-

grinding the bone to a suitable particle size in liquid nitrogen which is critical to the process depends on the mass of bone used, the size of the gross pieces, and the density of the bone. In any case, milling must be done in short pulses, for example, of 5 to 15 seconds for small quantities of bone powder (50 mg), and for not too long a total time. One must monitor the specific processes for the specific type of bone, the amount of bone, and so forth, by transmission electron microscopy to make certain that processing has not caused changes in either the size, shape, or physical and chemical characteristics of the crystals.

When these techniques are used with more calcified and mature bone, however, the yield of dispersed crystals is less than that obtained from the younger, less heavily mineralized bone. A second procedure with a higher yield of isolated, dispersed crystals free of collagen fibrils and other organic constituents as observed by electron microscopy, can be obtained even from mature, normally calcified bone. This technique was developed based on observations that both aggregates of isolated crystals free of collagen fibrils and particles composed of aggregates of collagen and apatite crystals could be dispersed and separated from one another when they were gently sonicated for short periods of time in cold, organic polar solvent such as 100% ethanol using low energy sonication. In this procedure, the diaphyses of fresh long bones cleaned of periosteum and cartilage are frozen in liquid nitrogen, cut into gross pieces, and cleaned endosteally. The pieces of bone are ground in a mill in very short 5 to 30 second pulsed bursts in liquid nitrogen to a particle size of up to approximately 200 microns, preferably 75 to 200 microns. Total grinding time depends on the size of the initial gross pieces and the density of the bone

(e.g., adult bovine bone compared with young chicken bone or small flexible fish bones).

b. Dissociation of bone matrix constituents.

The dehydration and chemical dissociation of the bone matrix constituents facilitates the disaggregation of the bone matrix by mechanical force, for example, by sonication.

The dried bone powder is suspended in an organic solvent such as ethanol precooled to just above its freezing temperature, in a container jacketed to maintain the low temperature of the solvent and bone powder. Particles are subjected to several low power sonications, each period lasting up to 10 minutes, depending on the volume of ethanol and the total mass of bone powder utilized, in order to disaggregate the dissociated bone matrix and the decomposed matrix and release single crystals and small aggregates of crystals. The ultrasonic disaggregation is carried out in a non-aqueous organic solvent such as ethanol, methanol, or other solvent with similar dielectric properties at 10°C or lower. This procedure can be repeated a number of times.

Suspending the bone particles in sodium dodecyl sulphate (SDS) in methanol or other non-aqueous solutions of organic detergents or dissociative compounds enhances disaggregation by decreasing forces between molecules in the matrix and thereby enhances the disaggregation of the matrix. Critical point drying using CO_2 after replacing absolute ethanol enhances the rate of dehydration and enhances the disaggregation of the matrix.

c. Decomposition of organic matrix constituents

The disaggregated materials are then treated with a polar non-aqueous solvent such as hydrazine (NH_2-NH_2) for varying periods of time at low temperatures, preferably less than 6°C, depending on the selection of solvent, most preferably between 2

and 4°C for hydrazine. Transmission electron microscopy of a low speed ultracentrifuged supernatant from relatively young bone treated with hydrazine reveals dispersed crystals of similar size and shape to the Ca-P crystals observed in bone as well as collagen fibril-crystal aggregates, which can then be removed by high speed centrifugation, leaving only the dispersed crystals free of collagen fibrils as observed by electron microscopy. Following low speed centrifugation, the crystals in the supernatant are separated from the solid bone particles, resuspended in a polar organic solvent such as ethanol (methanol being too polar) and thoroughly washed, centrifuged and filtered in cold ethanol or other equivalent solvent.

d. Disaggregation

Single crystals and small aggregates of crystals are released from the bone matrix following hydrazine treatment by washing in an organic solvent such as ethanol or methanol at a temperature of 10°C or less and dried by ether or ethyl acetate extraction.

e. Dispersion

The single crystals and small aggregates of crystals are treated with low power plasma ashing for short periods of time (five to ten minutes at 5 watts) and then dispersed in organic solvents such as ethanol, methanol or other solvents or similar dielectric properties. Treatment with low power plasma ashing for short period of time assures that the single crystals and small aggregates of crystals are dispersed into the solvent. Crystals and crystal aggregates released directly by plasma ashing of bone particles (equivalent to 10 to 50 watts, five hours) at a temperature of 0°C or less can also be dispersed in ethanol, methanol or equivalent solvents.

f. Separation of single crystals

Single crystals dispersed in ethanol, methanol or equivalent solvents remain in the supernatant when subjected to a force of under 1,000 g, whereas small aggregates of crystals are present in the pellet. After separating single crystals from aggregates, the single crystals are harvested by centrifugation at 30,000 g. The small crystal aggregates can then be processed through the disaggregation and separation procedures to obtain additional single crystals.

The final steps to remove the remaining organic matrix constituents are facilitated by the use of single crystals or very small amounts of small crystal aggregates. This technique permits the use of preparations containing principally single crystals which greatly enhances the final steps of matrix and solvent removal.

g. Removal of solvents

Additional low power plasma ashing completely removes solvents that may remain bound to the crystals. This procedure insures that the crystals are solvent free as well as free of organic matrix constituents. The crystals can also be dried by suspending and washing in 100% ethyl acetate several times, drying by passing dry N_2 gas over the crystals and vacuum evaporating.

Characterization of calcium-phosphate crystals obtained in initial separation.

After the first two steps, the crystals still have associated with them some residual organic constituents or breakdown products of the organic constituents, as shown by analytical chemical analyses, although electron microscopy reveals only the apatite crystals and no collagen fibrils or other organic components.

Hydrazine reaction with young bone followed by sonication in organic polar solvent yield isolated crystals free of collagen fibrils by electron

microscopy. The crystals are then thoroughly washed a number of times. As an additional step to remove the residual organic material remaining on the crystal surfaces, the crystals are suspended in 100% ethanol or equivalent organic polar solvent, with solid carbon dioxide, and treated by low energy plasma ashing (for small quantities of bone, approximately 5 mg, with low power defined as one watt or less, for 5 hours).

Chemical analysis, x-ray diffraction and Fourier transform infrared spectroscopy (FT-IR) of the crystals show no significant differences from analyses of whole, native bone, including the presence of carbonate and acid phosphate groups, and the failure to detect hydroxyl groups by fourier transform infrared spectroscopy and proton-nuclear magnetic resonance spectroscopy, signature characteristics of native, bone apatite crystals.

Applications for Purified Calcium-phosphate crystals:

Chromatographic Separations Applications.

The isolated bone crystals can be used to purify and isolate biological molecules such as proteins, glycoproteins, carbohydrates, and nucleotides, by standard chromatographic and other analytical and preparative technologies. Synthetic apatites are already used for chromatography but performance varies from batch to batch and from producer to producer, depending on how the synthetic apatites are made, sized, and so forth.

The Ca-P crystals isolated as described herein are more uniform in size, chemical composition, and structure from the synthetic apatites, and differ from synthetic apatites because they have certain specific functional groups such as carbonate and acid phosphate moieties in specific locations and at specific energy levels as well as small amounts of other ions, many of which are on the surfaces of the crystals and in the case of bone crystals, for example, no detectable

hydroxyl groups. Accordingly, they should provide more reproducible separations of specific components from a mixture of many components and also may be able to more selectively separate specific molecules originally present in the organic matrix of tissues such as bone and tooth with which they have specifically interacted in the native tissue as a result of these unique chemical and structural characteristics of the native bone crystals.

Therapeutic Applications.

The purified calcium-phosphate crystals isolated from bone are useful as an aid to induce and promote bone healing. Synthetic apatite crystals have been shown to be biocompatible, both *in vivo* and in bone cell (osteoblast cell) cultures (Cordoba, et al., "Effect of microcrystalline hydroxyapatite on bone marrow stromal cell osteogenesis", Thirty Ninth Annual Meeting, Orthopaedic Research Society, 1993). There is also experimental evidence that synthetic apatite preparations can act as osteoinductors, biologically active materials which induce cells to form bone, and osteoinductors, biologically active materials which facilitate and provide a scaffolding on which bone formation can spread and advance.

Accordingly, the crystals have a variety of therapeutic applications, either alone or in conjunction with other substances bound to the crystals such as, for example, other bone cell inducers and stimulators, including the general class of cytokines such as the TGF-ß superfamily of bone growth factors (Cytokines and Bone Metabolism, Gowen, ed (CRC press, 1992), the teachings of which are incorporated by reference herein, (available from Boehringer-Mannheim), the family of bone morphogenetic proteins, osteoinductors, and/or bone marrow or bone forming precursor cells, isolated using standard techniques. With reference to sources and amounts of

various materials that can be included with the crystals, see, for example, Glowacki, J., et al. "The role of osteocalcin in osteoclast differentiation" J Cellular Biochem 45:292-302 (1991); Ballock, T.T., et al. "Regulation of collagen expression in periosteal cells by three members of the TGF-B superfamily" Thirty Ninth Annual Meeting, Orthopaedic Research Society; 18,734 (1993); Ripamonti, U., et al. "Induction of bone in composites of osteogenin and porous hydroxyapatite in baboons" J. Plastic and Reconstructive Surg. 89:731-739 (1991); Ripamonti, U., et al. "Growth and morphogenetic factors in bone induction: role of osteogenin and related bone morphogenetic proteins" CRC Critical Reviews in Oral Biol. Med. 3:1-14 (1992); Ripamonti, U., et al. "Initiation of bone regeneration in baboons by osteogenin, a bone morphogenetic protein" Matrix; 12:40-55 (1992); Ripamonti, U., et al. "Xenogeneic osteogenin and demineralized bone matrices including human induced bone differentiation in athymic rats and baboons" Matrix 11:404-411 (1991); Cook, S.D., et al. "Restoration or large diaphyseal segmental defects in rabbits using recombinant human osteogenic protein (OP-1)" Combined meetings of Orthopaedic Research societies of USA, Japan and Canada 1, 66 (1991); Miyamoto, S., et al. "Trans-filter bone induction in monkeys by bone morphogenetic protein" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 99 (1993); Yasko, A.W., et al. "Comparison of recombinant human BMP-2 versus cancellous bone to heal segmental bone defects" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 100 (1993); Aspenberg, P., et al. "Bone morphogenetic protein induces bone in the squirrel monkey, but bone matrix does not" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 101 (1993); Iwasaki, M., et al. "Bone morphogenetic protein-2 stimulates osteogenesis

in high density culture of periosteum-derived cells" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 483 (1993); Cook, S.D., et al. "Recombinant human osteogenic protein-1 (rhOP-1) heals segmental long-bone defects in non-human primates" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 484 (1993); and Hunt, T.R., et al. "Healing of a segmental defect in the rat femur using a bone inducing agent (BIA) derived from a cultured human osteosarcoma cell line (SAOS-2)" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 489 (1993), the teachings of which are incorporated by reference herein. The crystals can also be mixed with antibiotics or chemotherapeutic agents.

The isolated calcium-phosphate crystals are useful as an aid in the healing of bone defects, fractures, and other similar situations, or in the treatment of local bone resorption such as occurs in periodontal tissue or in the fixation of prosthetic implanted teeth into the bony jaws, especially when combined with substances which inhibit or lessen bone resorption such as isoleukin 1-ß, for example, and osteoinductors such as the family of TGF-ß. The crystals can be applied as a cement, in combination with a binder, preferably a biodegradable polymeric matrix, although non-biodegradable polyacrylate and cyanoacrylates are also useful, or as a paste. They can be sprayed or otherwise applied to the surface of prosthetics prior to or at the time of implantation, using in combination with an adhesive applied to the surface of the implant. They can also be used as filler in gaps in the bone resulting from trauma, infection, or cancer, using other materials to serve as structural supports until the crystals are replaced by newly formed bone. See, for example, Aberman, H.M., et al. "Gap healing in a non-weight bearing dog model: effectiveness of a solution precipitated

apatite coating". Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 466 (1993).

A major complication of the use of artificial skeletal joints as implants and prosthetic teeth as implants in the loosening of artificial joint and tooth prostheses implanted into bone due to bony resorption about the implant. Examination of the surrounding tissues and prostheses has shown that this is due to the formation of cellular reactive connective tissue which forms after implantation and which incites resorption of the bone. This in turn is felt to be due for the most part to excessive micromotion between the implant and the living bone into which it is placed, presumably due to failure of sufficient interface appositional strength between the bone surface and the prosthesis. Recent work has also shown that when the appositional surfaces of the prosthesis are coated with apatite crystals, there is a significant increase in the appositional strength between the prosthetic surface and the implant and the surface of the bone which greatly diminishes the micromotion between the two components. See, for example, Bragdon, C.R., et al. "The histology of bone ingrowth at the implant/bone interface under known amounts of micromotion" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 468 (1993); Burke, D.W., et al. "Mechanical aspects of the bone-porous surface interface under known amounts of implant motion: an in vivo canine study" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 470 (1993); and Hollis, M.J., et al. "Effect of micromotion on ingrowth into porous coated implants" Thirty Ninth Annual Meeting, Orthopaedic Research Society 18, 472 (1993).

The crystals can be used in powdered form, shaped into blocks of ceramic, porous coatings, or mixed with other materials for use as coatings or

cements, using the methods and materials known to those skilled in this art. These are then useful for the manufacture of artificial prostheses, coatings on artificial joints or implanted prosthetic teeth and using the biological bone crystals embedded and combined with a biodegradable carrier. Components which induce bone formation and others which diminish bone resorption as well as antibiotics and other chemotherapeutic agents can be combined with the apatite crystals.

To date, there is no available detailed analysis of the intricate chemistry and structure of the synthetic apatites used to coat the prostheses. Although the methods of preparation of the synthetic apatites varies, they are generally formulated and applied to the metallic surfaces of the implants by plasma spraying which results not only in the formation of crystalline hydroxyapatites (but of varying chemical composition) and what has been term "amorphous" calcium-phosphates as well as calcium salts in addition to calcium-phosphate apatites. The specific chemical and physical characteristics of the native or natural calcium-phosphate crystals of bone isolated from bone, formulated to allow for increased bone formation (i.e. bone induction) and osteoinduction should not only permit a much increased interfacial appositional strength and bond between the bony surfaces and the implanted device (joint or tooth implant) but also allow a more bony bonding between the implant and the bone into which the implant is placed.

In all of these applications, care should be taken not to alter or damage the crystals, for example, by exposure to high temperatures and water.

Example 1: Preparation of Purified Bone Crystals

METHODS

1. Dissociation of bone matrix

Bone powder is reacted three times with absolute ethanol and three times with ether or ethyl acetate to dehydrate the bone matrix (10 ml/100 mg of bone powder). This treatment is followed by critical point drying and chemical dissociation. For the critical point drying, bone matrix is immersed in absolute ethanol, and is replaced with liquid CO_2 (10 ml/100 mg bone powder). The temperature is then slowly increased up to the critical point of CO_2 (31°C at 72.9 atmospheres). For the chemical dissociation of bone matrix, the bone particles are reacted with saturated sodium dodecl sulphate in methanol or other detergents or dissociative compounds in organic solvents at 20°C which enhances the subsequent disaggregation (10 ml/100 mg of bone powder) by decreasing the forces between organic molecules in the organic matrix.

2. Removal of organic matrix constituents:

Lipid is removed from the bone powder by reaction with methanol/chloroform (1:2), or ether or ethyl acetate treatment (10 ml/100 mg of bone powder) after reaction with absolute ethanol. This procedure is repeated three or more times.

Proteins and other organic matrix constituents are removed by three extractions of bone powder or of powdered crystal (single crystals and small aggregates of crystals) with hydrazine at low temperature (2-4°C). Each extraction is for a period of 12 hours followed by intermittent low power ultrasonication (270 watt/63 KHz, peak-output/frequency) for less than 3 minutes. It can be also removed by the repeated low power plasma ashing (5-10 watts for 5 hrs or crystal powder at 10-50 watts for 5 hrs.)

3. Disaggregation of bone matrix

Bone powder already dissociated physically or chemically as above is disaggregated in absolute ethanol (1 ml/100mg) by the force of low power ultrasonic force (270 watt/63 KHz, peak-output/frequency) at under 10°C which releases single crystals and small aggregates of single crystals.

4. Dispersion of single crystals

Low power (5 watt) plasma ashing treatment of the dissociated and/or disaggregated powder for short periods of time (5 minutes) at 0°C or lower. This makes it possible to disperse single crystals and small aggregates of crystals in solvents such as ethanol and methanol or other solvents of similar dielectrical properties.

5. Separation of single crystals from small aggregates of crystals.

Remove small aggregates of crystals dispersed in methanol or ethanol or other organic solvents (see above) from the suspension by centrifugation at 1,000g for 10 minutes. The single crystals which remain in the supernatant are then collected by ultracentrifugation at 30,000g for 30 minutes.

6. Washing and drying.

The bone powder or crystals are washed in absolute ethanol (10 ml/100 mg) three times and collected by centrifugation at 10,000 g. Samples are further washed with ether or ethyl acetate three times and dried in vacuum.

7. Removal of remaining solvents.

Dried crystals are treated with low power plasma ashing (5 watts for 10 minutes at 0°C or lower) to remove crystal bound solvents and any remaining organic matrix constituents.

We claim:

1. A composition of calcium-phosphate apatite crystals of the same chemical composition, structure, short range order, and index of crystallinity as the calcium-phosphate crystals present in bone selected from the group consisting of bone, cartilage, cementum, dentin, and enamel, essentially free of collagen fibrils.

2. The composition of claim 1 containing less than 25% of the dry total weight as organic material.

3. The composition of claim 2 containing less than 1% dry total weight as organic material.

4. The composition of claim 1 wherein the crystals are irregularly shaped, very thin plates whose rough average dimensions are approximately 10 to 50 angstroms in thickness, 30 to 150 angstroms in width, and 200 to 600 angstroms in length.

5. The composition of claim 1 further comprising adhesive or binder to form a calcium-phosphate crystalline cement or paste.

6. The composition of claim 1 applied to the surfaces of solid materials for implantation.

7. The composition of claim 1 formed into ceramic blocks for implantation into gaps or areas of bone resorption.

8. The composition of claim 1 further comprising a biologically active molecule selected from the group consisting of bone morphogenic proteins, cytokines, antibiotics, chemotherapeutic agents, bone marrow or bone progenitor cells, and substances that diminish bone resorption.

9. The composition of claim 1 produced by

forming clean bone particles into particles ranging in size of up to approximately 200 microns;

separating the calcium-phosphate crystals in the bone from the collagen fibrils in the bone by

sonication at low temperature in a non-aqueous solvent; and

removing the solvent and collagen fibrils from the calcium phosphate crystals.

10. The composition of claim 3 produced by

forming clean bone particles into particles ranging in size of up to approximately 200 microns;

separating the calcium-phosphate crystals in the bone from the collagen fibrils in the bone by sonication at low temperature in a non-aqueous solvent;

removing the solvent and collagen fibrils from the calcium phosphate crystals; and

removing the remaining organic material from the calcium phosphate crystals to form a composition containing less than 2% of the total dry weight as organic material.

11. The composition of claim 10 wherein the remaining organic material is removed by reaction with an inorganic non-aqueous solvent under conditions equivalent to reaction with hydrazine at low temperatures overnight.

12. The composition of claim 10 wherein the remaining organic material is removed by plasma ashing.

13. The composition of claim 1 wherein the bone is mature, mineralized bone.

14. The composition of claim 1 wherein the crystals are derived from a material selected from the group consisting of bone, dentin, enamel, cementum, and cartilage.

15. A method for isolating calcium-phosphate apatite crystals from bone comprising the steps of

forming clean bone particles into particles ranging in size of up to approximately 200 microns, where the bone is selected from the group consisting of bone, cartilage, cementum, dentin, and enamel;

separating the calcium-phosphate crystals in the bone from the collagen fibrils in the bone by sonication at low temperature in a non-aqueous solvent; and

removing the solvent and collagen fibrils from the calcium phosphate crystals.

16. The method of claim 15 further comprising

removing the remaining organic material from the calcium phosphate crystals to form crystals containing less than 2% of the total dry weight as organic material.

17. The method of claim 16 wherein the remaining organic material is removed by reaction with an polar non-aqueous solvent under conditions equivalent to reaction with hydrazine at low temperatures overnight.

18. The method of claim 16 wherein the remaining organic material is removed by plasma ashing at low temperatures.

19. The method of claim 15 wherein the bone is mature, mineralized bone.

20. The method of claim 15 wherein the crystals are derived from a material selected from the group consisting of bone is selected from the group consisting of bone, cartilage, cementum, dentin, and enamel.

21. The method of claim 15 wherein the non-aqueous solvent is anhydrous 100% ethanol and the calcium-phosphate crystals are separated from the collagen fibrils by centrifugation at low speeds.

22. The method of claim 15 wherein the crystals are irregularly shaped, very thin plates whose rough average dimensions are approximately 10 to 50 angstroms in thickness, 30 to 150 angstroms in width, and 200 to 600 angstroms in length.

23. The method of claim 15 further comprising adding to the crystals an adhesive or binder to form a calcium-phosphate crystalline cement or paste.

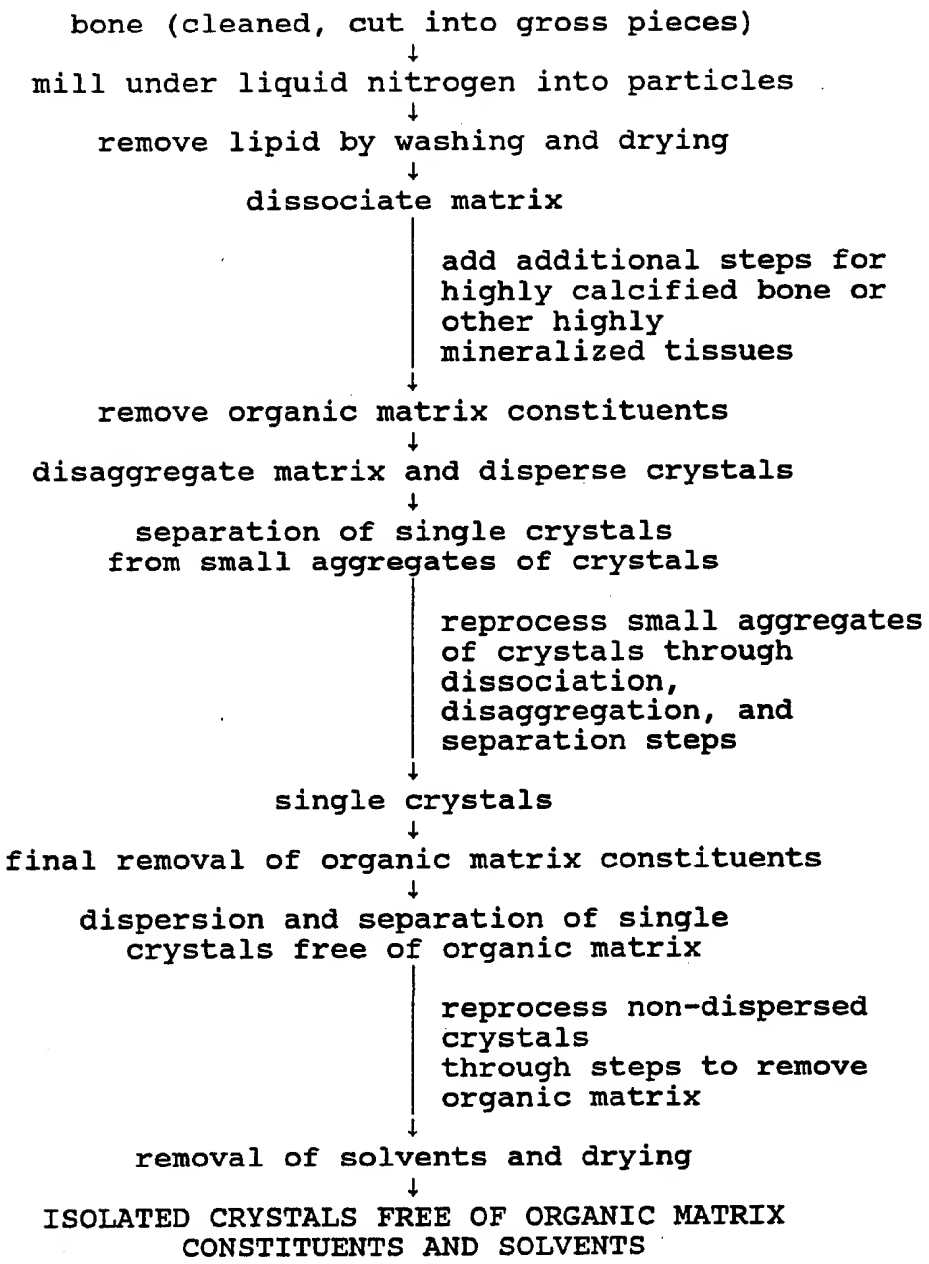
24. The method of claim 15 further comprising applying the crystals to the surfaces of solid materials for implantation.

25. The method of claim 15 further comprising forming the crystals into ceramic blocks for implantation into gaps or areas of bone resorption.

26. The method of claim 15 further comprising adding to the crystals a biologically active molecule selected from the group consisting of bone morphogenic proteins, cytokines, antibiotics, chemotherapeutic agents, and bone marrow or bone progenitor cells.

27. The method of claim 15 wherein the process is applied to coral to remove the organic material from the calcium carbonate forming the coral structure.

FIGURE 1


bone (cleaned, cut into gross pieces)
mill under liquid nitrogen into particles
remove lipid by washing and drying
dissociate matrix
add additional steps for highly calcified bone or other highly mineralized tissues
remove organic matrix constituents
disaggregate matrix and disperse crystals
separation of single crystals from small aggregates of crystals
reprocess small aggregates of crystals through dissociation, disaggregation, and separation steps
single crystals
final removal of organic matrix constituents
dispersion and separation of single crystals free of organic matrix
reprocess non-dispersed crystals through steps to remove organic matrix
removal of solvents and drying
ISOLATED CRYSTALS FREE OF ORGANIC MATRIX CONSTITUENTS AND SOLVENTS

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 94/03214

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C01B25/32 A61L27/00 C01F11/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C01B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 9020, Derwent Publications Ltd., London, GB; AN 90-152280 & JP,A,2 097 409 (TAIYO FISHERY KK ET AL.) 10 April 1990 see abstract ---	
A	CHEMICAL ABSTRACTS, vol. 115, no. 8, 26 August 1991, Columbus, Ohio, US; abstract no. 74640y, page 176 ; see abstract	
A	& JP,A,03 109 210 (TAIYO KAGAKU KOGYO CO., LTD.) 9 May 1991 --- -/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 July 1994	-4. 0894

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 9235, Derwent Publications Ltd., London, GB; AN 92-288770 & JP,A,4 198 007 (KANEBO LTD ET AL.) 17 July 1992 see abstract	
A	& PATENT ABSTRACTS OF JAPAN vol. 16, no. 524 (C-1000)28 October 1992 & JP,A,04 198 007 (KANEBO LTD ET AL.) 17 July 1992 see abstract	
A	JOURNAL OF DENTAL RESEARCH vol. 67, no. 9 , September 1988 , WASHINGTON,D.C.,US pages 1229 - 1234 T. SAKAE ET AL. cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. nal Application No
PCT/US 94/03214

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP-A-03109210	09-05-91	NONE	